



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷: A61K 39/395, C07K 16/28, C12N 15/13	A1	(11) International Publication Number: WO 00/18437 (43) International Publication Date: 6 April 2000 (06.04.00)
(21) International Application Number: PCT/US99/22421 (22) International Filing Date: 28 September 1999 (28.09.99) (30) Priority Data: 60/102,100 28 September 1998 (28.09.98) US (71) Applicants (for all designated States except US): SMITHK-LINE BEECHAM CORPORATION [US/US]; One Franklin Plaza, Philadelphia, PA 19103 (US). SMITHK-LINE BEECHAM PLC [GB/GB]; New Horizons Court, Brentford, Middlesex TW8 9EP (GB). (72) Inventors; and (75) Inventors/Applicants (for US only): HOLMES, Stephen, D. [GB/GB]; New Frontiers Science Park South, Third Avenue, Hariow, Essex CM19 5AW (GB). ERICKSON-MILLER, Connie, L. [US/US]; 608 Brainerd Place, Exton, PA 19341 (US). WINKLER, James, D. [US/US]; 701 Hartranft Avenue, Fort Washington, PA 19034 (US). (74) Agents: BAUMEISTER, Kirk et al.; SmithKline Beecham Corporation, Corporate Intellectual Property, UW2220, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406-0939 (US).		(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: TIE2 ANTAGONIST ANTIBODIES (57) Abstract Tie2 receptor antagonist antibodies and their use in inhibiting angiogenesis are disclosed.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

TIE2 ANTAGONIST ANTIBODIES

Field of the Invention

This invention relates to antagonist monoclonal antibodies (mAb) that bind
5 to the Tie2 receptor and to the use of such antibodies for therapeutic purposes.

Background of the Invention

Chronic proliferative diseases are often accompanied by profound
angiogenesis, which can contribute to or maintain an inflammatory and/or
10 proliferative state, or which leads to tissue destruction through the invasive
proliferation of blood vessels. (Folkman, EXS 79:1-8, 1997; Folkman, Nature
Medicine 1:27-31, 1995; Folkman and Shing, J. Biol. Chem. 267:10931, 1992).

Angiogenesis or neovascularisation is the process of development of new or
replacement blood vessels. It is a necessary and normal process by which the
15 vasculature is established in the embryo. Angiogenesis does not occur, in general, in
most normal adult tissues, exceptions being sites of ovulation, menses and wound
healing.

Many diseases, however, are characterized by persistent and unregulated
angiogenesis. For instance, in arthritis, new capillary blood vessels invade the joint
20 and destroy cartilage (Colville-Nash and Scott, Ann. Rheum. Dis., 51, 919, 1992). In
diabetes (and in many different eye diseases), new vessels invade the macula or
retina or other ocular structures, and may cause blindness (Brooks *et al.*, Cell, 79,
1157, 1994). The process of atherosclerosis has been linked to angiogenesis
(Kahlon *et al.*, Can. J. Cardiol. 8, 60, 1992). Tumor growth and metastasis have
25 been found to be angiogenesis-dependent (Folkman, Cancer Biol. 3, 65, 1992;
Denekamp, Br. J. Rad. 66, 181, 1993; Fidler and Ellis, Cell, 79, 185, 1994).

The recognition of the involvement of angiogenesis in major diseases has
been accompanied by research to identify and develop angiogenesis inhibitors.
Angiogenesis occurs in many stages and inhibitors are generally classified in
30 response to discrete targets in the angiogenesis cascade, such as activation of
endothelial cells by an angiogenic signal; synthesis and release of degradative
enzymes; endothelial cell migration; proliferation of endothelial cells; and formation
of capillary tubules. Literature reports indicate that inhibitors of angiogenesis,
working by diverse mechanisms, are beneficial in diseases such as cancer and
35 metastasis (O'Reilly *et al.*, Cell, 79, 315, 1994; Ingber *et al.*, Nature, 348, 555,
1990), ocular diseases (Friedlander *et al.*, Science, 270, 1500, 1995), arthritis

(Peacock *et al.*, J. Exp. Med. 175, 1135, 1992; Peacock *et al.*, Cell. Immun. 160, 178, 1995) and hemangioma (Taraboletti *et al.*, J. Natl. Cancer Inst. 87, 293, 1995).

In recent years, it has become clear that while angiogenesis is a complex, multicellular phenomena, specific ligands and their receptors play a key role. In particular, a combination of studies suggest that the Tie2 receptor and its ligand are important in angiogenesis.

Tie2 receptor has been located in endothelial cells of all forming blood vessels and in the endocardium of mouse embryos (Korhonen *et al.*, Blood 80:2548-2555, 1992). Selective patterns of expression in endothelial cells during embryonic development has also been demonstrated (Schlaeger *et al.*, Development 121:1089-1098, 1995).

In adult tissues, Tie mRNA cannot be observed in skin, except at sites of active wound healing where the proliferating capillaries in the granulation tissue contain abundant Tie mRNA (Korhonen *et al.*, Blood 80:2548-2555, 1992). Further, Tie receptor is expressed in the vascular endothelium of metastasizing melanomas (Kaipainen *et al.*, Cancer Res. 54:6571-6577, 1994). While Tie receptor expression is down-regulated in the established vasculature, it is up-regulated in angiogenesis that occurs in the ovary during ovulation, in wounds and in tumor (breast, melanoma and renal cell carcinoma) vasculature, consistent with prevailing views that angiogenesis in the adult borrows from embryonic angiogenic mechanisms.

Homozygous mice with a Tie2 knockout, or carrying a transgene encoding a "dominant-negative" Tie2 receptor, confirmed that the Tie2 receptor is critical for embryonic development (Dumont *et al.*, Genes Dev. 8:1897-1909, 1994; Sato *et al.*, Nature 376:70-74, 1995). Embryonic death in these mice occurred due to vascular insufficiency and there were dramatically reduced numbers of endothelial cells. Vasculogenesis -- that is the differentiation of endothelial cells and the *in situ* formation of vessels -- appeared relatively normal in mice lacking Tie2. The subsequent sprouting and remodeling resulting in formation of vessel branches (angiogenesis) was drastically reduced in the Tie2 mutant mice embryos. This lack of sprouting and angiogenesis resulted in substantial growth retardation, particularly of the brain, neural tube and heart, resulting in lack of viability. These results exemplify the critical importance of Tie2 in angiogenesis. This is significant, as angiogenesis is regulated by a number of growth factors. Interestingly, Flk1 (VEGF receptor) knockout mice exhibit embryo lethal defects in vasculogenesis, that occur earlier than those of Tie2 disruption. Disruption of the Tie1 receptor yields a much different, and later, defective phenotype; the mouse embryo dies late in development due to hemorrhage resulting from defective integrity of an otherwise well-formed

vasculature. Taken together, these studies suggest that the VEGF/Flk1 and Tie systems operate in sequential fashion, with Tie2 having a critical role in angiogenesis.

Recently, two ligands for the Tie2 receptor have been reported.

- 5 Angiopoietin-1 binds and induces the tyrosine phosphorylation of Tie2 and its expression *in vivo* is in close proximity with developing blood vessels (Davis *et al.*, Cell 87:1161-1169,1996). Mice engineered to lack Angiopoietin-1 display angiogenic deficits reminiscent of those previously seen in mice lacking Tie2 receptors, demonstrating that Angiopoietin-1 is a primary physiologic ligand for
10 Tie2 and that Tie2 has critical *in vivo* angiogenic actions (Suri *et al.*, Cell 87:1171-1180, 1996). Angiopoietin-2 was identified by homology screening and shown to be a naturally occurring antagonist for Tie2 receptors. Transgenic overexpression of Angiopoietin-2 disrupts blood vessel formation in the mouse embryo (Maisonpierre *et al.*, Science 277:55-60, 1997). Together, these results support a role for Tie2
15 receptors in angiogenesis.

- The Tie1 and Tie2 receptors are single-transmembrane, tyrosine kinase receptors (Tie stands for Tyrosine kinase receptors with immunoglobulin and EGF homology domains). They are the only receptor tyrosine kinases, other than those
20 receptors for VEGF, that are largely restricted to endothelial cells in their expression. Both have been cloned and reported by several groups (Dumont *et al.*, Oncogene 8:1293-1301, 1993; Partanen *et al.*, Mol. Cell Biol. 12:1698-1707, 1992; Sato *et al.*, Proc. Natl. Acad. Sci. USA 90:9355-9358, 1993).

- The Tie receptors are proteins of approximately 125 kDa, with a single
25 putative transmembrane region. The extracellular domain of these receptors is uniquely divided into three regions that have a pattern of cysteine expression found in EGF-like domains; two regions that have some weak homology to and structural characteristics of immunoglobulin-like domains; and three regions with homology to the fibronectin III repeat structure. The intracellular portion of Tie2 is most closely related (~40 % identity) to the kinase domains of FGF-R1, PDGF-R and c-kit. The
30 intracellular portions of Tie2 contain all of the features of tyrosine kinases, including a GXGXXG ATP binding site consensus sequence and typical tyrosine kinase motifs (*i.e.*, HRDLAARN and DFGL).

- Based upon the importance of Tie2 receptors in angiogenesis, inhibition of Tie2 activity is predicted to interrupt angiogenesis, providing disease-specific
35 therapeutic effects. Recently, Lin *et al.* (J. Clin. Invest. 100:2072-2078, 1997) has shown that exogenously administered soluble Tie2 receptor inhibited angiogenesis and cancer growth in animal models. Clearly, there is a need to develop high

affinity, potent antagonist antibodies to the Tie2 receptor which will have sufficient activity to work *in vivo* at therapeutically acceptable concentrations.

Tie2 receptors are expressed on hematopoietic progenitors, including CD34+ cells, megakaryocyte progenitors and megakaryocyte-derived cell lines (Kukk *et al.*, Brit J Haematol. 98:195-203, 1997; Batard *et al.*, Blood 87:2212-2220, 1996). The Tie2 receptor has homology to other hematopoietic growth factor receptors such as c-kit (Chabot *et al*, Nature 335:88, 1988; Yarden *et al*, EMBO J 6:3341, 1987) and flk-2 (Matthews *et al*, Cell 65:1143, 1991). The expression of Tie2 decreased on more mature hematopoietic cells, although it was maintained on a significant fraction of cells during megakaryocytic differentiation (Batard). These data, taken together, suggest that Tie2 is a receptor for a hematopoietic growth factor acting on early progenitors and during differentiation of the megakaryocytic lineage.

Summary of the Invention

One aspect of the present invention is a Tie2 receptor antagonist antibody having the identifying characteristics of monoclonal antibody 12H8 or 6A3.

Another aspect of the invention is an antibody comprising a heavy chain variable region polypeptide as set forth in SEQ ID NO: 2 and a light chain variable region polypeptide as set forth in SEQ ID NO: 4 and isolated polynucleotides encoding them.

Another aspect of the invention is a heavy chain CDR having an amino acid sequence as set forth in SEQ ID NO: 5, 6 or 7.

Another aspect of the invention is a light chain CDR having an amino acid sequence as set forth in SEQ ID NO: 8, 9 or 10.

Another aspect of the invention is a hybridoma having the identifying characteristics of cell line 12H8 or 6A3.

Another aspect of the invention is a method for inhibiting angiogenesis in a animal comprising administering an effective dose of an Tie2 receptor antagonist antibody having the identifying characteristics of monoclonal antibody 12H8 or 6A3.

Another aspect of the invention is a method for expansion of marrow cells transfected with a gene for gene therapy comprising treating the marrow cells with a Tie2 receptor antagonist antibody.

Another aspect of the invention is a method for expansion of cord blood, marrow or peripheral blood cells for transplant comprising treating the cells with a Tie2 receptor antagonist antibody.

Another aspect of the invention is a method to enhance the survival of hematopoietic progenitor cells in a mammal comprising treating the cells with a Tie2 receptor antagonist antibody.

- 5 Another aspect of the invention is a method to increase the proliferation of megakaryocyte cells or megakaryocyte progenitor cells to increase platelet numbers in a mammal comprising treating the cells with a Tie2 receptor antagonist antibody.

Detailed Description of the Invention

- 10 All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as though fully set forth.

As used herein, the term "inhibiting angiogenesis" means decreasing the development of new or replacement blood vessels (neovascularization).

- 15 As used herein, the term "antagonist activity" refers to the activity of an antibody that binds to Tie2 receptor and inhibits angiogenesis.

As used herein, the term "treating" and derivatives thereof means prophylactic, palliative or therapeutic therapy.

- 20 The present invention provides a variety of antibodies, including altered antibodies and fragments thereof directed against Tie2 receptor, which are characterized by antagonist activity. Exemplary Tie2 receptor antagonist antibodies are the murine monoclonal antibodies 12H8 or 6A3.

- "Antibodies" refers to immunoglobulins which can be prepared by conventional hybridoma techniques, phage display combinatorial libraries, immunoglobulin chain shuffling and humanization techniques. Also included are 25 fully human monoclonal antibodies. As used herein, "antibody" also includes "altered antibody" which refers to a protein encoded by an altered immunoglobulin coding region, which may be obtained by expression in a selected host cell. Such altered antibodies are engineered antibodies (*e.g.*, chimeric or humanized antibodies) or antibody fragments lacking all or part of an immunoglobulin constant region, *e.g.*, 30 Fv, Fab, Fab' or F(ab')₂ and the like. These antibody products are useful in therapeutic and pharmaceutical compositions for treating angiogenic and proliferative (including inflammatory) diseases and other conditions which are caused by excessive or inappropriate angiogenesis. Exemplary angiogenic diseases include diabetic retinopathy and macular degeneration. Exemplary proliferative 35 diseases include cancer, arthritis, psoriasis and atherosclerosis. Alternatively, the antibodies of the invention can be coupled to toxins, antiproliferative drugs or

radionuclides to kill cells in areas of excessive Tie2 receptor expression and/or angiogenesis.

"Altered immunoglobulin coding region" refers to a nucleic acid sequence encoding an altered antibody of the invention. When the altered antibody is a complementarity determining region-grafted (CDR-grafted) or humanized antibody, the sequences that encode the CDRs from a non-human immunoglobulin are inserted into a first immunoglobulin partner comprising human variable framework sequences. Optionally, the first immunoglobulin partner is operatively linked to a second immunoglobulin partner.

"First immunoglobulin partner" refers to a nucleic acid sequence encoding a human framework or human immunoglobulin variable region in which the native (or naturally-occurring) CDR-encoding regions are replaced by the CDR-encoding regions of a donor antibody. The human variable region can be an immunoglobulin heavy chain, a light chain (or both chains), an analog or functional fragments thereof. Such CDR regions, located within the variable region of antibodies (immunoglobulins) can be determined by known methods in the art. For example Kabat *et al.* in "Sequences of Proteins of Immunological Interest", 4th Ed., U.S. Department of Health and Human Services, National Institutes of Health (1987) disclose rules for locating CDRs. In addition, computer programs are known which are useful for identifying CDR regions/structures.

"Second immunoglobulin partner" refers to another nucleotide sequence encoding a protein or peptide to which the first immunoglobulin partner is fused in frame or by means of an optional conventional linker sequence (*i.e.*, operatively linked). Preferably, it is an immunoglobulin gene. The second immunoglobulin partner may include a nucleic acid sequence encoding the entire constant region for the same (*i.e.*, homologous, where the first and second altered antibodies are derived from the same source) or an additional (*i.e.*, heterologous) antibody of interest. It may be an immunoglobulin heavy chain or light chain (or both chains as part of a single polypeptide). The second immunoglobulin partner is not limited to a particular immunoglobulin class or isotype. In addition, the second immunoglobulin partner may comprise part of an immunoglobulin constant region, such as found in a Fab, or F(ab)₂ (*i.e.*, a discrete part of an appropriate human constant region or framework region). Such second immunoglobulin partner may also comprise a sequence encoding an integral membrane protein exposed on the outer surface of a host cell, *e.g.*, as part of a phage display library, or a sequence encoding a protein for analytical or diagnostic detection, *e.g.*, horseradish peroxidase, β -galactosidase, etc.

The terms Fv, Fc, Fd, Fab, Fab' or F(ab')₂ are used with their standard meanings. See, e.g., Harlow *et al.* in "Antibodies A Laboratory Manual", Cold Spring Harbor Laboratory, (1988).

As used herein, an "engineered antibody" describes a type of altered
5 antibody, i.e., a full-length synthetic antibody (e.g., a chimeric or humanized
antibody as opposed to an antibody fragment) in which a portion of the light and/or
heavy chain variable domains of a selected acceptor antibody are replaced by
analogous parts from one or more donor antibodies which have specificity for the
selected epitope. For example, such molecules may include antibodies characterized
10 by a humanized heavy chain associated with an unmodified light chain (or chimeric
light chain), or vice versa. Engineered antibodies may also be characterized by
alteration of the nucleic acid sequences encoding the acceptor antibody light and/or
heavy variable domain framework regions in order to retain donor antibody binding
specificity. These antibodies can comprise replacement of one or more CDRs
15 (preferably all) from the acceptor antibody with CDRs from a donor antibody
described herein.

A "chimeric antibody" refers to a type of engineered antibody which contains
a naturally-occurring variable region (light chain and heavy chains) derived from a
donor antibody in association with light and heavy chain constant regions derived
20 from an acceptor antibody.

A "humanized antibody" refers to a type of engineered antibody having its
CDRs derived from a non-human donor immunoglobulin, the remaining
immunoglobulin-derived parts of the molecule being derived from one or more
human immunoglobulins. In addition, framework support residues may be altered to
25 preserve binding affinity. See, e.g., Queen *et al.*, Proc. Natl Acad Sci USA, **86**,
10029-10032 (1989), Hodgson *et al.*, Bio/Technology, **9**, 421 (1991). Furthermore,
as described herein, additional residues may be altered to preserve the antagonist
activity of the donor antibody.

The term "donor antibody" refers to a monoclonal or recombinant antibody
30 which contributes the nucleic acid sequences of its variable regions, CDRs or other
functional fragments or analogs thereof to a first immunoglobulin partner, so as to
provide the altered immunoglobulin coding region and resulting expressed altered
antibody with the antigenic specificity and neutralizing activity characteristic of the
donor antibody. Donor antibodies suitable for use in this invention is a murine
35 antagonist monoclonal antibody designated as 12H8 and 6A3.

The term "acceptor antibody" refers to monoclonal or recombinant antibodies
heterologous to the donor antibody, which contributes all, or a portion, of the nucleic

acid sequences encoding its heavy and/or light chain framework regions and/or its heavy and/or light chain constant regions or V region subfamily consensus sequences to the first immunoglobulin partner. Preferably, a human antibody is the acceptor antibody.

- 5 "CDRs" are defined as the complementarity determining region amino acid sequences of an antibody which are the hypervariable regions of immunoglobulin heavy and light chains. See, e.g., Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 4th Ed., U.S. Department of Health and Human Services, National Institutes of Health (1987). There are three heavy chain and three light
10 chain CDRs or CDR regions in the variable portion of an immunoglobulin. Thus, "CDRs" as used herein refers to all three heavy chain CDRs, or all three light chain CDRs or both all heavy and all light chain CDRs, if appropriate.

- CDRs provide the majority of contact residues for the binding of the antibody to the antigen or epitope. CDRs of interest in this invention are derived
15 from donor antibody variable heavy and light chain sequences, and include analogs of the naturally occurring CDRs, which analogs share or retain the same antigen binding specificity and/or antagonist ability as the donor antibody from which they were derived, yet may exhibit increased affinity for the antigen. An exemplary process for obtaining analogs is affinity maturation by means of phage display
20 technology as reviewed by Hoogenboom, *Trends in Biotechnology* 15, 62-70 (1997); Barbas *et al.*, *Trends in Biotechnology* 14, 230-234 (1996); and Winter *et al.*, *Ann. Rev. Immunol.* 12, 433-455 (1994) and described by Irving *et al.*, *Immunotechnology* 2, 127-143 (1996).

- By "sharing the antigen binding specificity or antagonist ability" is meant,
25 for example, that although mAb 12H8 or 6A3 may be characterized by a certain level of antagonist activity, a CDR encoded by a nucleic acid sequence of 12H8 or 6A3 in an appropriate structural environment may have a lower or higher activity. It is expected that CDRs of 12H8 or 6A3 in such environments will nevertheless recognize the same epitope(s) as 12H8 or 6A3.

- 30 A "functional fragment" is a partial heavy or light chain variable sequence (e.g., minor deletions at the amino or carboxy terminus of the immunoglobulin variable region) which retains the same antigen binding specificity and/or antagonist ability as the antibody from which the fragment was derived.

- An "analog" is an amino acid sequence modified by at least one amino acid,
35 wherein said modification can be chemical or a substitution or a rearrangement of a few amino acids (*i.e.*, no more than 10) and corresponding nucleic acid sequences, which modification permits the amino acid sequence to retain the biological

characteristics, *e.g.*, antigen specificity and high affinity, of the unmodified sequence. Exemplary nucleic acid analogs include silent mutations which can be constructed, via substitutions, to create certain endonuclease restriction sites within or surrounding CDR-encoding regions.

- 5 Analogues may also arise as allelic variations. An "allelic variation or modification" is an alteration in the nucleic acid sequence encoding the amino acid or peptide sequences of the invention. Such variations or modifications may be due to degeneracy in the genetic code or may be deliberately engineered to provide desired characteristics. These variations or modifications may or may not result in
10 alterations in any encoded amino acid sequence.

- The term "effector agents" refers to non-protein carrier molecules to which the altered antibodies, and/or natural or synthetic light or heavy chains of the donor antibody or other fragments of the donor antibody may be associated by
conventional means. Such non-protein carriers can include conventional carriers
15 used in the diagnostic field, *e.g.*, polystyrene or other plastic beads, polysaccharides, *e.g.*, as used in the BIAcore (Pharmacia) system, or other non-protein substances useful in the medical field and safe for administration to humans and animals. Other effector agents may include a macrocycle, for chelating a heavy metal atom or radioisotopes. Such effector agents may also be useful to increase the half-life of the
20 altered antibodies, *e.g.*, polyethylene glycol.

- For use in constructing the antibodies, altered antibodies and fragments of this invention, a non-human species such as bovine, ovine, monkey, chicken, rodent (*e.g.*, murine and rat) may be employed to generate a desirable immunoglobulin upon presentation with human Tie2 receptor or a peptide epitope therefrom.
25 Conventional hybridoma techniques are employed to provide a hybridoma cell line secreting a non-human mAb to the Tie2 receptor. Such hybridomas are then screened for binding and antagonist activity as described in the Examples section. Alternatively, fully human mAbs can be generated by techniques known to those skilled in the art and used in this invention.

- 30 Exemplary antagonist mAbs of the present invention is mAb 12H8 and 6A3, murine antibodies which can be used for the development of a chimeric or humanized molecule. The 12H8 and 6A3 mAbs are characterized by antagonist activity on human Tie2 receptor and subsequent blocking of the receptor's signal transduction resulting in inhibition of angiogenesis. These mAbs are produced by the
35 hybridoma cell lines 12H8 and 6A3, respectively.

 The present invention also includes the use of Fab fragments or F(ab')₂ fragments derived from mAbs directed against Tie2 receptor as bivalent fragments.

These fragments are useful as agents having antagonist activity at the Tie2 receptor. A Fab fragment contains the entire light chain and amino terminal portion of the heavy chain. An F(ab')₂ fragment is the fragment formed by two Fab fragments bound by disulfide bonds. The mAbs 12H8 and 6A3 and other similar high affinity
5 antibodies provide sources of Fab fragments and F(ab')₂ fragments which can be obtained by conventional means, *e.g.*, cleavage of the mAb with the appropriate proteolytic enzymes, papain and/or pepsin, or by recombinant methods. These Fab and F(ab')₂ fragments are useful themselves as therapeutic, prophylactic or
10 diagnostic agents, and as donors of sequences including the variable regions and CDR sequences useful in the formation of recombinant or humanized antibodies as described herein.

The Fab and F(ab')₂ fragments can be constructed via a combinatorial phage library (see, *e.g.*, Winter *et al.*, *Ann. Rev. Immunol.*, 12:433-455 (1994)) or via immunoglobulin chain shuffling (see, *e.g.*, Marks *et al.*, *Bio/Technology*, 10:779-783
15 (1992)), wherein the Fd or V_H immunoglobulin from a selected antibody (*e.g.*, 12H8 or 6A3) is allowed to associate with a repertoire of light chain immunoglobulins, V_L (or V_K), to form novel Fabs. Conversely, the light chain immunoglobulin from a selected antibody may be allowed to associate with a repertoire of heavy chain immunoglobulins, V_H (or Fd), to form novel Fabs. Tie2 receptor antagonist Fabs
20 can be obtained by allowing the Fd of mAb 12H8 or 6A3 to associate with a repertoire of light chain immunoglobulins. Hence, one is able to recover neutralizing Fabs with unique sequences (nucleotide and amino acid) from the chain shuffling technique.

The mAb 12H8 or 6A3 may contribute sequences, such as variable heavy
25 and/or light chain peptide sequences, framework sequences, CDR sequences, functional fragments, and analogs thereof, and the nucleic acid sequences encoding them, useful in designing and obtaining various altered antibodies which are characterized by the antigen binding specificity of the donor antibody.

The nucleic acid sequences of this invention, or fragments thereof, encoding
30 the variable light chain and heavy chain peptide sequences are also useful for mutagenic introduction of specific changes within the nucleic acid sequences encoding the CDRs or framework regions, and for incorporation of the resulting modified or fusion nucleic acid sequence into a plasmid for expression. For example, silent substitutions in the nucleotide sequence of the framework and CDR-
35 encoding regions can be used to create restriction enzyme sites which facilitate insertion of mutagenized CDR and/or framework regions. These CDR-encoding regions can be used in the construction of the humanized antibodies of the invention.

The nucleic and amino acid sequences of the 12H8 heavy chain variable region is listed in SEQ ID NO: 1. The CDR amino acid sequences from this region are listed in SEQ ID Nos: 5, 6 and 7.

5 The nucleic and amino acid sequences of the 12H8 light chain variable region listed in SEQ ID NO: 3. The CDR amino acid sequences from this region are listed in SEQ ID Nos: 8, 9 and 10.

Taking into account the degeneracy of the genetic code, various coding sequences may be constructed which encode the variable heavy and light chain amino acid sequences and CDR sequences of the invention as well as functional
10 fragments and analogs thereof which share the antigen specificity of the donor antibody. The isolated nucleic acid sequences of this invention, or fragments thereof, encoding the variable chain peptide sequences or CDRs can be used to produce altered antibodies, e.g., chimeric or humanized antibodies or other engineered antibodies of this invention when operatively combined with a second
15 immunoglobulin partner.

It should be noted that in addition to isolated nucleic acid sequences encoding portions of the altered antibody and antibodies described herein, other such nucleic acid sequences are encompassed by the present invention, such as those complementary to the native CDR-encoding sequences or complementary to the
20 modified human framework regions surrounding the CDR-encoding regions. Useful DNA sequences include those sequences which hybridize under stringent hybridization conditions to the DNA sequences. See, T. Maniatis *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory (1982), pp. 387-389. An example of one such stringent hybridization condition is hybridization at
25 4XSSC at 65°C, followed by a washing in 0.1XSSC at 65°C for one hour. Alternatively, an exemplary stringent hybridization condition is 50% formamide, 4XSSC at 42°C. Preferably, these hybridizing DNA sequences are at least about 18 nucleotides in length, i.e., about the size of a CDR.

Altered immunoglobulin molecules can encode altered antibodies which
30 include engineered antibodies such as chimeric antibodies and humanized antibodies. A desired altered immunoglobulin coding region contains CDR-encoding regions that encode peptides having the antigen specificity of an Tie2 receptor antibody, preferably a high-affinity antagonist antibody such as provided by the present invention, inserted into a first immunoglobulin partner such as a human
35 framework or human immunoglobulin variable region.

Preferably, the first immunoglobulin partner is operatively linked to a second immunoglobulin partner. The second immunoglobulin partner is defined above,

and may include a sequence encoding a second antibody region of interest, for example an Fc region. Second immunoglobulin partners may also include sequences encoding another immunoglobulin to which the light or heavy chain constant region is fused in frame or by means of a linker sequence. Engineered antibodies directed
5 against functional fragments or analogs of the Tie2 receptor may be designed to elicit enhanced binding with the same antibody.

The second immunoglobulin partner may also be associated with effector agents as defined above, including non-protein carrier molecules, to which the second immunoglobulin partner may be operatively linked by conventional means.

10 Fusion or linkage between the second immunoglobulin partners, *e.g.*, antibody sequences, and the effector agent may be by any suitable means, *e.g.*, by conventional covalent or ionic bonds, protein fusions, or hetero-bifunctional cross-linkers, *e.g.*, carbodiimide, glutaraldehyde and the like. Such techniques are known in the art and are described in conventional chemistry and biochemistry texts.

15 Additionally, conventional linker sequences which simply provide for a desired amount of space between the second immunoglobulin partner and the effector agent may also be constructed into the altered immunoglobulin coding region. The design of such linkers is well known to those of skill in the art.

In addition, signal sequences for the molecules of the invention may be
20 modified by techniques known to those skilled in the art to enhance expression.

A preferred altered antibody contains a variable heavy and/or light chain peptide or protein sequence having the antigen specificity of mAb 12H8 or 6A3, *e.g.*, the V_H and V_L chains. Still another desirable altered antibody of this invention is characterized by the amino acid sequence containing at least one, and preferably
25 all of the CDRs of the variable region of the heavy and/or light chains of the murine antibody molecule 12H8 or 6A3 with the remaining sequences being derived from a human source, or a functional fragment or analog thereof.

In a further embodiment, the altered antibody of the invention may have attached to it an additional agent. For example, recombinant DNA technology may
30 be used to produce an altered antibody of the invention in which the Fc fragment or CH2 CH3 domain of a complete antibody molecule has been replaced by an enzyme or other detectable molecule, *i.e.*, a polypeptide effector or reporter molecule. Other additional agents include toxins, antiproliferative drugs and radionuclides.

The second immunoglobulin partner may also be operatively linked to a non-
35 immunoglobulin peptide, protein or fragment thereof heterologous to the CDR-containing sequence having antigen specificity to the Tie2 receptor. The resulting protein may exhibit both antigen specificity and characteristics of the non-

immunoglobulin upon expression. That fusion partner characteristic may be, *e.g.*, a functional characteristic such as another binding or receptor domain or a therapeutic characteristic if the fusion partner is itself a therapeutic protein or additional antigenic characteristics.

- 5 Another desirable protein of this invention may comprise a complete antibody molecule, having full length heavy and light chains or any discrete fragment thereof, such as the Fab or F(ab')₂ fragments, a heavy chain dimer or any minimal recombinant fragments thereof such as an F_v or a single-chain antibody (SCA) or any other molecule with the same specificity as the selected donor mAb,
10 *e.g.*, the 12H8 or 6A3 mAb. Such protein may be used in the form of an altered antibody or may be used in its unfused form.

- Whenever the second immunoglobulin partner is derived from an antibody different from the donor antibody, *e.g.*, any isotype or class of immunoglobulin framework or constant regions, an engineered antibody results. Engineered
15 antibodies can comprise immunoglobulin constant regions and variable framework regions from one source, *e.g.*, the acceptor antibody, and one or more (preferably all) CDRs from the donor antibody, *e.g.*, the 12H8 or 6A3 mAb. In addition, alterations, *e.g.*, deletions, substitutions, or additions, of the acceptor mAb light and/or heavy variable domain framework region at the nucleic acid or amino acid levels, or the
20 donor CDR regions may be made in order to retain donor antibody antigen binding specificity.

- Such engineered antibodies are designed to employ one (or both) of the variable heavy and/or light chains of the Tie2 receptor mAb (optionally modified as described) or one or more of the heavy or light chain CDRs. The engineered
25 antibodies of the invention exhibit antagonist activity.

- Such engineered antibodies may include a humanized antibody containing the framework regions of a selected human immunoglobulin or subtype or a chimeric antibody containing the human heavy and light chain constant regions fused to the Tie2 receptor mAb functional fragments. A suitable human (or other
30 animal) acceptor antibody may be one selected from a conventional database, *e.g.*, the KABAT® database, Los Alamos database, and Swiss Protein database, by homology to the nucleotide and amino acid sequences of the donor antibody. A human antibody characterized by a homology to the V region frameworks of the donor antibody or V region subfamily consensus sequences (on an amino acid basis)
35 may be suitable to provide a heavy chain variable framework region for insertion of the donor CDRs. A suitable acceptor antibody capable of donating light chain variable framework regions may be selected in a similar manner. It should be noted

that the acceptor antibody heavy and light chains are not required to originate from the same acceptor antibody.

Preferably, the heterologous framework and constant regions are selected from human immunoglobulin classes and isotypes, such as IgG (subtypes 1 through 4), IgM, IgA, and IgE. IgG1, k and IgG4, k are preferred. Particularly preferred is IgG 4, k. Most particularly preferred is the IgG4 subtype variant containing the mutations S228P and L235E (PE mutation) in the heavy chain constant region which results in reduced effector function. This IgG4 subtype variant is known herein as IgG4PE. See U.S. Patent Nos. 5, 624,821 and 5,648,260.

The acceptor antibody need not comprise only human immunoglobulin protein sequences. For instance, a gene may be constructed in which a DNA sequence encoding part of a human immunoglobulin chain is fused to a DNA sequence encoding a non-immunoglobulin amino acid sequence such as a polypeptide effector or reporter molecule.

A particularly preferred humanized antibody contains CDRs of 12H8 or 6A3 mAb inserted onto the framework regions of a selected human antibody sequence. For antagonist humanized antibodies, one, two or preferably three CDRs from the 12H8 or 6A3 antibody heavy chain and/or light chain variable regions are inserted into the framework regions of the selected human antibody sequence, replacing the native CDRs of the human antibody.

Preferably, in a humanized antibody, the variable domains in both human heavy and light chains have been engineered by one or more CDR replacements. It is possible to use all six CDRs, or various combinations of less than the six CDRs. Preferably all six CDRs are replaced. It is possible to replace the CDRs only in the human heavy chain, using as light chain the unmodified light chain from the human acceptor antibody. Still alternatively, a compatible light chain may be selected from another human antibody by recourse to conventional antibody databases. The remainder of the engineered antibody may be derived from any suitable acceptor human immunoglobulin.

The engineered humanized antibody thus preferably has the structure of a natural human antibody or a fragment thereof, and possesses the combination of properties required for effective therapeutic use, *e.g.*, treatment of angiogenic diseases such as diabetic retinopathy and macular degeneration or treatment of proliferative diseases, such as cancer, arthritis, psoriasis and atherosclerosis.

It will be understood by those skilled in the art that an engineered antibody may be further modified by changes in variable domain amino acids without necessarily affecting the specificity and high affinity of the donor antibody (*i.e.*, an

analog). It is anticipated that heavy and light chain amino acids may be substituted by other amino acids either in the variable domain frameworks or CDRs or both. These substitutions could be supplied by the donor antibody or consensus sequences from a particular subgroup.

- 5 In addition, the constant region may be altered to enhance or decrease selective properties of the molecules of this invention. For example, dimerization, binding to Fc receptors, or the ability to bind and activate complement (see, *e.g.*, Angal *et al.*, *Mol. Immunol.*, 30, 105-108 (1993), Xu *et al.*, *J. Biol. Chem.*, 269, 3469-3474 (1994), Winter *et al.*, EP 307434-B).

- 10 An altered antibody which is a chimeric antibody differs from the humanized antibodies described above by providing the entire non-human donor antibody heavy chain and light chain variable regions, including framework regions, in association with human immunoglobulin constant regions for both chains. It is anticipated that chimeric antibodies which retain additional non-human sequence relative to
15 humanized antibodies of this invention may elicit a significant erythropoietic response in humans. Such antibodies are useful in the prevention of and for treating angiogenic diseases such as diabetic retinopathy and macular degeneration or treatment of proliferative diseases, such as cancer, arthritis, psoriasis and atherosclerosis.

- 20 Preferably, the variable light and/or heavy chain sequences and the CDRs of mAb 12H8 or 6A3 or other suitable donor mAbs and their encoding nucleic acid sequences, are utilized in the construction of altered antibodies, preferably humanized antibodies, of this invention, by the following process. The same or similar techniques may also be employed to generate other embodiments of this
25 invention.

- A hybridoma producing a selected donor mAb, *e.g.*, the murine antibody 12H8 or 6A3, is conventionally cloned and the DNA of its heavy and light chain variable regions obtained by techniques known to one of skill in the art, *e.g.*, the techniques described in Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*,
30 2nd edition, Cold Spring Harbor Laboratory (1989). The variable heavy and light regions containing at least the CDR-encoding regions and those portions of the acceptor mAb light and/or heavy variable domain framework regions required in order to retain donor mAb binding specificity, as well as the remaining immunoglobulin-derived parts of the antibody chain derived from a human
35 immunoglobulin, are obtained using polynucleotide primers and reverse transcriptase. The CDR-encoding regions are identified using a known database and by comparison to other antibodies.

A mouse/human chimeric antibody may then be prepared and assayed for binding ability. Such a chimeric antibody contains the entire non-human donor antibody V_H and V_L regions, in association with human Ig constant regions for both chains.

- 5 Homologous framework regions of a heavy chain variable region from a human antibody are identified using computerized databases, *e.g.*, KABAT®, and a human antibody characterized by a homology to the V region frameworks of the donor antibody or V region subfamily consensus sequences (on an amino acid basis) to 12H8 or 6A3 is selected as the acceptor antibody. The sequences of synthetic
10 heavy chain variable regions containing the CDR-encoding regions within the human antibody frameworks are designed with optional nucleotide replacements in the framework regions to incorporate restriction sites. This designed sequence is then synthesized using long synthetic oligomers. Alternatively, the designed sequence can be synthesized by overlapping oligonucleotides, amplified by
15 polymerase chain reaction (PCR), and corrected for errors. A suitable light chain variable framework region can be designed in a similar manner.

- A humanized antibody may be derived from the chimeric antibody, or preferably, made synthetically by inserting the donor mAb CDR-encoding regions from the heavy and light chains appropriately within the selected heavy and light
20 chain framework. Alternatively, a humanized antibody of the invention may be prepared using standard mutagenesis techniques. Thus, the resulting humanized antibody contains human framework regions and donor mAb CDR-encoding regions. There may be subsequent manipulation of framework residues. The resulting humanized antibody can be expressed in recombinant host cells, *e.g.*, COS,
25 CHO or myeloma cells.

- A conventional expression vector or recombinant plasmid is produced by placing these coding sequences for the altered antibody in operative association with conventional regulatory control sequences capable of controlling the replication and expression in, and/or secretion from, a host cell. Regulatory sequences include
30 promoter sequences, *e.g.*, CMV or Rous Sarcoma virus promoter, and signal sequences, which can be derived from other known antibodies. Similarly, a second expression vector can be produced having a DNA sequence which encodes a complementary antibody light or heavy chain. Preferably, this second expression vector is identical to the first except with respect to the coding sequences and
35 selectable markers, in order to ensure, as much as possible, that each polypeptide chain is functionally expressed. Alternatively, the heavy and light chain coding sequences for the altered antibody may reside on a single vector.

A selected host cell is co-transfected by conventional techniques with both the first and second vectors (or simply transfected by a single vector) to create the transfected host cell of the invention comprising both the recombinant or synthetic light and heavy chains. The transfected cell is then cultured by conventional techniques to produce the engineered antibody of the invention. The humanized antibody which includes the association of both the recombinant heavy chain and/or light chain is screened from culture by an appropriate assay such as ELISA or RIA. Similar conventional techniques may be employed to construct other altered antibodies and molecules of this invention.

Suitable vectors for the cloning and subcloning steps employed in the methods and construction of the compositions of this invention may be selected by one of skill in the art. For example, the pUC series of cloning vectors, such as pUC19, which is commercially available from supply houses, such as Amersham or Pharmacia, may be used. Additionally, any vector which is capable of replicating readily, has an abundance of cloning sites and selectable genes (*e.g.*, antibiotic resistance) and is easily manipulated may be used for cloning. Thus, the selection of the cloning vector is not a limiting factor in this invention.

Similarly, the vectors employed for expression of the engineered antibodies according to this invention may be selected by one of skill in the art from any conventional vector. The vectors also contain selected regulatory sequences (such as CMV or Rous Sarcoma virus promoters) which direct the replication and expression of heterologous DNA sequences in selected host cells. These vectors contain the above-described DNA sequences which code for the engineered antibody or altered immunoglobulin coding region. In addition, the vectors may incorporate the selected immunoglobulin sequences modified by the insertion of desirable restriction sites for ready manipulation.

The expression vectors may also be characterized by genes suitable for amplifying expression of the heterologous DNA sequences, *e.g.*, the mammalian dihydrofolate reductase gene (DHFR). Other preferable vector sequences include a poly A signal sequence, such as from bovine growth hormone (BGH) and the betaglobin promoter (betaglopro). The expression vectors useful herein may be synthesized by techniques well known to those skilled in this art.

The components of such vectors, *e.g.*, replicons, selection genes, enhancers, promoters, signal sequences and the like, may be obtained from commercial or natural sources or synthesized by known procedures for use in directing the expression and/or secretion of the product of the recombinant DNA in a selected host. Other appropriate expression vectors of which numerous types are known in

the art for mammalian, bacterial, insect, yeast and fungal expression may also be selected for this purpose.

The present invention also encompasses a cell line transfected with a recombinant plasmid containing the coding sequences of the engineered antibodies or altered immunoglobulin molecules thereof. Host cells useful for the cloning and other manipulations of these cloning vectors are also conventional. However, most desirably, cells from various strains of *E. coli* are used for replication of the cloning vectors and other steps in the construction of altered antibodies of this invention.

Suitable host cells or cell lines for the expression of the engineered antibody or altered antibody of the invention are preferably mammalian cells such as CHO, COS, a fibroblast cell (e.g., 3T3) and myeloid cells, and more preferably a CHO or a myeloid cell. Human cells may be used, thus enabling the molecule to be modified with human glycosylation patterns. Alternatively, other eukaryotic cell lines may be employed. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Sambrook *et al.*, *supra*.

Bacterial cells may prove useful as host cells suitable for the expression of the recombinant Fabs of the present invention (see, e.g., Plückthun, A., *Immunol. Rev.*, 130, 151-188 (1992)). However, due to the tendency of proteins expressed in bacterial cells to be in an unfolded or improperly folded form or in a non-glycosylated form, any recombinant Fab produced in a bacterial cell would have to be screened for retention of antigen binding ability. If the molecule expressed by the bacterial cell was produced in a properly folded form, that bacterial cell would be a desirable host. For example, various strains of *E. coli* used for expression are well-known as host cells in the field of biotechnology. Various strains of *B. subtilis*, *Streptomyces*, other bacilli and the like may also be employed.

Where desired, strains of yeast cells known to those skilled in the art are also available as host cells, as well as insect cells, e.g. *Drosophila* and *Lepidoptera*, and viral expression systems. See, e.g. Miller *et al.*, *Genetic Engineering*, 8, 277-298, Plenum Press (1986) and references cited therein.

The general methods by which the vectors of the invention may be constructed, the transfection methods required to produce the host cells of the invention, and culture methods necessary to produce the altered antibody of the invention from such host cell are all conventional techniques. Likewise, once produced, the altered antibodies of the invention may be purified from the cell culture contents according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis

and the like. Such techniques are within the skill of the art and do not limit this invention.

Yet another method of expression of the humanized antibodies may utilize expression in a transgenic animal, such as described in U. S. Patent No. 4,873,316.

- 5 This relates to an expression system using the animal's casein promoter which when transgenically incorporated into a mammal permits the female to produce the desired recombinant protein in its milk.

Once expressed by the desired method, the engineered antibody is then examined for *in vitro* activity by use of an appropriate assay. Presently, surface
10 plasmon resonance is employed to assess qualitative and quantitative binding of the engineered antibody to Tie2 receptor. Additionally, other *in vitro* assays such as the Matrigel vascularization model may also be used to determine antagonist activity prior to subsequent human clinical studies performed to evaluate the persistence of the engineered antibody in the body despite the usual clearance mechanisms.

- 15 Following the procedures described for humanized antibodies prepared from 12H8 or 6A3, one of skill in the art may also construct humanized antibodies from other donor antibodies, variable region sequences and CDR peptides described herein. Engineered antibodies can be produced with variable region frameworks potentially recognized as "self" by recipients of the engineered antibody.
20 Modifications to the variable region frameworks can be implemented to effect increases in antigen binding and antagonist activity without appreciable increased immunogenicity for the recipient. Such engineered antibodies may effectively treat a human for ischemic diseases such as myocardial infarction or cerebral stroke or treatment of vascular insufficiency diseases, such as diabetes. Such antibodies may
25 also be useful in the diagnosis of those conditions.

This invention also relates to a method for enhancing angiogenesis in an mammal, particularly a human, which comprises administering an effective dose of an Tie2 receptor monoclonal antibody having antagonist activity. The mAb can include one or more of the antibodies or altered antibodies described herein or
30 fragments thereof.

The therapeutic response induced by the use of the molecules of this invention is produced by the binding to the Tie2 receptor and the subsequent antagonist activity of the angiogenic process. Thus, the molecules of the present invention, when in preparations and formulations appropriate for therapeutic use, are
35 highly desirable for persons susceptible to or experiencing angiogenic diseases such as diabetic retinopathy or macular degeneration or proliferative diseases, such as cancer, arthritis, psoriasis or atherosclerosis.

This invention also relates to a method for expansion of marrow cells transfected with a gene for gene therapy comprising treating the marrow cells with a Tie2 receptor antagonist antibody. Preferably, the expansion is *ex vivo*.

5 This invention also relates to a method for expansion of cord blood, marrow or peripheral blood cells for transplant comprising treating the cells with a Tie2 receptor antagonist antibody. Preferably, the expansion is *ex vivo*.

This invention also relates to a method to enhance the survival of hematopoietic progenitor cells in a mammal comprising treating the cells with a Tie2 receptor antagonist antibody.

10 This invention also relates to a method to increase the proliferation of megakaryocyte cells or megakaryocyte progenitor cells to increase platelet numbers in a mammal comprising treating the cells with a Tie2 receptor antagonist antibody.

The mAb used in the methods of the invention can include one or more of the antibodies or altered antibodies described herein or fragments thereof.
15 Preferably, the Tie2 receptor antagonist antibody used in the methods of the invention has the identifying characteristics of mAb 12H8 or 6A3.

The altered antibodies, antibodies and fragments thereof of this invention may also be used in conjunction with other antibodies, particularly human mAbs reactive with other markers (epitopes) responsible for the condition against which
20 the engineered antibody of the invention is directed.

The Tie2 receptor antagonist antibodies of the invention can be formulated into pharmaceutical compositions and administered in the same manner as described for mature proteins. See, e.g., International Patent Application, Publication No. WO90/02762 (Mar. 22 1990). Generally, these compositions contain a
25 therapeutically effective amount of an antagonist antibody of this invention and an acceptable pharmaceutical carrier. Suitable carriers are well known to those of skill in the art and include, for example, saline. Alternatively, such compositions may include conventional delivery systems into which protein of the invention is incorporated. Optionally, these compositions may contain other active ingredients.

30 The therapeutic agents of this invention may be administered by any appropriate internal route, and may be repeated as needed, e.g., as frequently as one to three times daily for between 1 day to about three weeks to once per week or once biweekly. Preferably, the antagonist antibody is administered less frequently than is the ligand, when it is used therapeutically. The dose and duration of treatment
35 relates to the relative duration of the molecules of the present invention in the human circulation, and can be adjusted by one of skill in the art depending upon the condition being treated and the general health of the patient.

As used herein, the term "pharmaceutical" includes veterinary applications of the invention. The term "therapeutically effective amount" refers to that amount of a receptor antagonist antibody, which is useful for alleviating a selected condition. These therapeutic compositions of the invention may be administered to mimic the effect of the normal receptor ligand.

The mode of administration of the therapeutic agent of the invention may be any suitable route which delivers the agent to the host. The altered antibodies, antibodies, engineered antibodies, and fragments thereof, and pharmaceutical compositions of the invention are particularly useful for parenteral administration, *i.e.*, subcutaneously, intramuscularly, intravenously or intranasally.

Therapeutic agents of the invention may be prepared as pharmaceutical compositions containing an effective amount of the engineered (*e.g.*, humanized) antibody of the invention as an active ingredient in a pharmaceutically acceptable carrier. In the compositions of the invention, an aqueous suspension or solution containing the engineered antibody, preferably buffered at physiological pH, in a form ready for injection is preferred. The compositions for parenteral administration will commonly comprise a solution of the engineered antibody of the invention or a cocktail thereof dissolved in an pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be employed, *e.g.*, 0.4% saline, 0.3% glycine and the like. These solutions are sterile and generally free of particulate matter. These solutions may be sterilized by conventional, well known sterilization techniques (*e.g.*, filtration). The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, etc. The concentration of the antibody of the invention in such pharmaceutical formulation can vary widely, *i.e.*, from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., according to the particular mode of administration selected.

Thus, a pharmaceutical composition of the invention for intramuscular injection could be prepared to contain 1 mL sterile buffered water, and between about 1 ng to about 100 mg, *e.g.* about 50 ng to about 30 mg or more preferably, about 5 mg to about 25 mg, of an engineered antibody of the invention. Similarly, a pharmaceutical composition of the invention for intravenous infusion could be made up to contain about 250 ml of sterile Ringer's solution, and about 1 mg to about 30 mg and preferably 5 mg to about 25 mg of an engineered antibody of the invention. Actual methods for preparing parenterally administrable compositions are well known or will be apparent to those skilled in the art and are described in more detail

in, for example, "Remington's Pharmaceutical Science", 15th ed., Mack Publishing Company, Easton, Pennsylvania.

It is preferred that the therapeutic agent of the invention, when in a pharmaceutical preparation, be present in unit dose forms. The appropriate
5 therapeutically effective dose can be determined readily by those of skill in the art. To effectively treat anemia in a human or other animal, one dose of approximately 0.01 mg to approximately 20 mg per kg body weight of a protein or an antibody of this invention should be administered parenterally, preferably *i.v.* or *i.m.* Such dose
10 may, if necessary, be repeated at appropriate time intervals selected as appropriate by a physician during the response period.

The present invention will now be described with reference to the following specific, non-limiting examples.

Example 1

Preparation and Screening of Tie2 Antagonist Monoclonal Antibodies

Mice (F1 hybrids of Balb/c and C57BL/6) were immunised subcutaneously with recombinant Tie2 extracellular domain-Fc fusion in RIBI adjuvant and boosted with the same. Alternatively, mice were immunized using Tie2 receptor-Fc fusion DNA and boosted with protein in RIBI adjuvant. A splenectomy was performed 3-4 days following the final immunization. Mouse spleen cells were used to prepare hybridomas by standard procedures, (Zola, H.Ed., Monoclonal Antibodies, CRC Press Inc. (1987)). Positive hybridomas were cloned by the limiting dilution method.

Immunoassay

To determine the specificity of the anti-Tie2 mAbs generated, 96-well plates were coated with Tie2-Fc and blocked. All the following incubations were performed in a shaker-incubator at RT. After washing the wells, Tie2-Fc or assay buffer and mAb / hybridoma supernatants were added in the presence of 20ug/ml human IgG1 (to eliminate anti-Fc mAbs) and incubated for 60 min. After washing the wells, Eu³⁺ labelled anti-mouse antibody in assay buffer was added for 60 min, the wells washed and then enhancer (Wallac) was added and incubated for 5 min at RT and the fluorescence measured. All positive hybridomas showed binding to Tie2.

Selection of Antibodies by BIAcore binding properties

BIAcore was used to select hybridomas and primary clones that bind to the external domain of the wild type Tie2 receptor. Antibodies were assessed for their ability to bind to Tie2-Fc by surface plasmon resonance using a BIAcore instrument. Rabbit anti-mouse IgG Fc specific antibody was immobilized on a sensor chip surface and mAb was injected, the refractive index units (RU; the RU are a direct measure of the amount of each protein which can bind) recorded, followed by injections of Tie2-Fc or IgG and record RU. The surface was regenerated with an injection of 15ul 0.1M phosphoric acid. The above was repeated on a goat anti-human IgG Fc specific sensor chip surface, i.e. sequential addition of Tie2-Fc or IgG and monoclonal antibody. Two hybridoma supernatants showed the presence of high-affinity antibodies. The hybridoma cell lines and antibodies produced were designated 12H8 and 6A3.

Purification of Mabs

Monoclonal antibodies 12H8 and 6A3 were purified by protein-A chromatography per the manufacturer's instructions from the selected hybridoma supernatants. Mabs were >95% pure by SDS-PAGE.

Affinity Measurements of Monoclonal Antibody

The affinity of the purified mAbs was measured in the BIAcore. Using a flow rate of 10ul/min, the mAb (diluted in HBS buffer) was injected over a rabbit anti-mouse IgG Fe surface, followed by buffer flow and the RU recorded. Tie2-Fc diluted in HBS buffer was then injected for 120s followed by buffer flow for 240s and regeneration of the sensor chip surface with an injection of 15 ul 0.1 M phosphoric acid. BIAcore software was used for association and dissociation-phase analysis. The murine monoclonal antibodies bound to soluble monomeric Tie2 receptor. The on-rates (k_{ass}) and off-rates (k_{diss}) were calculated. Together, these yield a calculated equilibrium constant (K_D) of <0.1 nM for mAb 12H8 and 0.1 nM for mAb 6A3.

Example 2

Functional Screening of Tie2 Antagonist Monoclonal Antibodies

Whole-cell activity assay for the Tie2 Receptor

HEL cells (ATCC # TIB180) are cultured at between 1 and 5×10^5 ml in RPMI-1640 medium supplemented with 2 mM glutamine and 10% FBS as a suspension culture. Sixteen to thirty-six hours prior to an experiment, the necessary number of cells are passaged into 0.5% FBS/RPMI medium. On the day of an experiment, cells are harvested and resuspended at a density of $0.5 - 1.0 \times 10^7$ cells/ml in 0.5% FBS RPMI and seeded at 2-3 ml/well in six well plates.

Alternatively, Human Umbilical Vein Endothelial Cells (HUVECs) (Cell Systems – Kirkland, WA) may be used for the assay. HUVECs between passages 2 and 12, are plated at 2×10^5 and 1×10^6 cells per well in a six well plate in CS-C medium supplemented with 10% serum and endothelial cell growth factor (Cell Systems). After 24 hours the media is changed to CS-C medium supplemented only with 2% serum, and the cells are cultured overnight and used for assay the following day.

Cells are treated with ligand or antibody at appropriate concentrations for 10 minutes (exact time of exposure and concentration of ligand must be determined empirically for the experimental conditions). The contents of the wells are mixed

- briefly on a rocker (approx 30 seconds) and then incubated at 37°C. For each experiment there are appropriate controls run, such that there is at least one control for every gel (e.g. for a ten lane gel there will be up to eight experimental treatments, one control and one lane for markers). Appropriate controls depend on activities analyzed. For agonist effects, an untreated control is used for a base-line comparison. For antagonists or inhibitors of phosphorylation, a culture stimulated with an equivalent agonist (e.g. conditioned medium from cultured rheumatoid fibroblasts) alone, is used.

- At the end of the 5-15 minute incubation period the plate is placed on ice.
- HEL cells are harvested by pipet. Media from HUVECs is removed, replaced with cold PBS, and the cells mechanically released from the plate surface with a policeman or cell scraper. The cells are spun down at 4° and the medium aspirated. The cell pellet is washed with cold PBS, centrifuged again, the PBS supernatant drawn off and the tubes/cell pellets submerged in a dry ice/ethanol bath. The cell pellet is removed to ice and allowed to thaw slightly. The pellet is resuspended in 500 µl of lysis buffer (RIPA lysis buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS+ inhibitors: 1 mM Sodium Orthovanadate, 1 mM Sodium Fluoride, 1 mM EDTA,+ protease inhibitors Sigma Mammalian cell protease inhibitor cocktail (#P8340) or a mix of PMSF, 1 mM aprotinin and Leupeptin - 10 µg / ml). The suspension is sonicated for 5 pulses at a medium setting and returned to ice. The homogenate is centrifuged to remove unlysed cells and the supernatants are transferred to microfuge tubes and kept on ice.

- The phosphorylation state of the Tie2 receptor is determined by immunoprecipitation of the Tie2 receptor, isolation by electrophoresis and detection by anti-phosphotyrosine Ab, as detailed below (Harlow, E., and Lane, D.P., Antibodies - a Laboratory Manual, Cold Spring Harbor Laboratory Press: New York, 1988.). An alternative assay was done, using antibody against SH-PTP2 to precipitated Tie2 receptors. Approximately seven ug of Tie2 antibody (TIE2 polyclonal antibody, Santa Cruz, Biotechnology Cat.# sc-324) are added to each lysate and each sample is incubated for 1 hour while mixing at 4°C. Twenty ul of a protein A or proteinG agarose slurry is added and the samples incubated for an additional hour while mixing at 4°C. The agarose/antibody complexes are pelleted for 2' at 2000-5000 rpm and 4°C in a microcentrifuge. The supernatant is carefully aspirated and the pellet is resuspended in 1ml cold lysis buffer and again centrifuged. This wash step is repeated an additional two times. After the last aspiration, 40 ul 1X SDS-PAGE sample buffer (Laemli)+ 2.5% 2-mercaptoethanol is added. The sample is vortexed and boiled for 3 minutes. Thirty ul are run on a

7.5% SDS/polyacrylamide gel. The gel is then transferred to a nitrocellulose or PVDF membrane as per the manufacturer's instructions for Western blotting.

The blots are washed with PBS 0.05% tween-20 and then blocked with 5% Non-fat dry milk / PBS/tween for 1 hour at room temperature. The blots are then incubated with 1 ug/ml anti-phosphotyrosine antibody (e.g Santa Cruz Biotech #SC508 or Upstate Biotech #05-321)-in PBS/0.05% tween for 1 hour. The blot is then washed 4 times with PBS/tween for 5 minutes each. The blot is incubated with an anti-mouse-HRP conjugate secondary antibody at the dilution recommended by the manufacturer, in PBS/tween for 1 hour. The blot is washed in PBS/tween, 4 times for 5 minutes each. After the last wash, the blot is developed by the ECL method (Amersham) or some equivalent.

After generating a satisfactory image, the blot is washed in 0.05% Tween 20-PBS for 5 minutes, and then antibody is stripped using 100 mM 2-mercaptoethanol-2% SDS-62.5 mM Tris-HCL, pH6.8 for 30 minutes at 50°C with occasional agitation. The blot is again washed in 0.05% Tween 20-PBS. In preparation for a second probing, the blots are blocked with 5% nonfat milk PBS/tween for 1 hour at room temperature, then incubated with 200 ng/ml anti-Tie2 antibody-3% nonfat milk PBS/tween for 1 hour. The blot is washed 4 times with PBS/tween for 5 minutes each, then incubated with an anti-rabbit-HRP antibody conjugate, diluted to the manufacturer's specifications in PBS/tween for 1 hour. After the blot is washed 4 times with PBS/tween for 5 minutes each, the image is developed by the ECL method.

Using a densitometer or graphics program (e.g. ImageQuant – Molecular Dynamics), each blot is scanned. The Tie-2 band is isolated and "boxed out" for each lane. The pixel volume or comparable measure for each sample is analyzed, for both Tie-2 staining and its corresponding phosphotyrosine staining. Also, an appropriate background region of the same dimensions is determined for each sample. After adjusting for background, phosphorylation is expressed as the ratio of phosphotyrosine staining, relative to the sample's corresponding Tie-2 staining.

The results indicated that mAb 12H8 consistently reduced RSF-conditioned medium induced phosphorylation of the Tie2 receptor between 60 and 100% at a maximum concentration of 50 ug/ml. Further, mAb 12H8 caused a reduction in the association of the Tie-2 receptor with the signalling protein SH-PTP2 over a range of 1ug/ml to 50ug/ml.

Survival Effects on Primitive Bone Marrow Cells

The light density cells of human bone marrow were removed from a Ficoll density gradient. CD34+ cells were separated by the MiniMACCs column (Miltenyi Biotech; Auburn, CA) and plated at 1×10^5 c/ml in 1 ml cultures in X-Vivo medium without serum.

The Tie2 antagonist mAb 6A3, was added to the cultures at 0, 0.1, 1 and 10 ug/ml and the cultures were incubated at 37°C, 5% CO₂ for 7 days. The cells were harvested, counted and analyzed for expression of CD34 by flow cytometry.

The antagonist Tie2 mAb, 6A3, enhanced the survival of cells from CD34+ purified cells. There were also an increased number of cells expressing the CD34 marker under antibody-treated conditions, suggesting that these cells did not undergo differentiation and lose this marker of more primitive cells.

A Tie2 antagonist antibody enhanced survival of primitive bone marrow cells and prevented differentiation of the cells. Accordingly, these antibodies would be useful for expansion of marrow cells transfected with a gene for gene therapy or expansion of cord blood, marrow or peripheral blood cells for transplant. Preferably, the expansion would be *ex vivo*. Determination of the amount of antibody used to treat cells is within the purview of one ordinarily skilled in the art. Treatment of a mammal with Tie2 mAb would increase the number of hematopoietic progenitors resulting in multi-lineage increases in blood cells and platelets.

Growth of UT7 (GM-CSF) Cells

Megakaryocytes, their progenitors and megakaryoblastic leukemia cells, such as UT7, express Tie2 receptors. A GM-CSF-dependent cell line UT7(GM-CSF) was plated at 5×10^4 cells/ml in Iscove's Modified Dulbecco's Medium with 10% FCS. GM-CSF was used at either 10 ng/ml (optimal) or 5 ng/ml (sub-optimal) and Tie2 antagonist mAb 6A3 at 1 ug/ml. At 24, 48 and 72 hours a sample of cells was removed from culture and counted.

The antagonist mAb 6A3 alone did not have an effect on the growth of the cytokine-dependent hematopoietic cell line UT7 (GM-CSF). However, the 6A3 mAb synergized with suboptimal amounts of GM-CSF to increase UT7 (GM-CSF) cell number at 24, 48 and 72 hrs. Treatment of a mammal with a Tie2 receptor mAb would increase the number of megakaryocyte progenitors, thereby increasing the number of platelets. Determination of the amount of antibody used to treat cells is within the purview of one ordinarily skilled in the art.

Example 3

Cloning and Sequencing of anti-TIE2 kinase receptor monoclonal antibody

Light and Heavy Chain cDNAs

5 The amino acid sequences of the light- and heavy-chain N- termini were determined for the anti-TIE2 kinase receptor mAb 12H8 by means of protein microsequencing methodology. Pyroglutamic acid blocked N- termini were first deblocked enzymatically using pyroglutamate aminopeptidase.

Total 12H8 hybridoma RNA was purified, reverse transcribed and PCR amplified. For the heavy chain, the RNA/DNA hybrid was PCR amplified using a mouse IgG CH1-specific primer and a degenerate primer based on the N-terminal protein sequence. Similarly, for the light chain, the RNA/DNA hybrid was PCR amplified using a mouse C kappa primer and a degenerate primer based on the N-terminal protein sequence. PCR products of the appropriate size, *i.e.*, ~350 were cloned into a plasmid vector, and sequenced by a modification of the Sanger method. In each case the sequence of VH and Vk clones were compared to generate a consensus 12H8 heavy chain variable region sequence (SEQ ID NOs: 1 and 2) and consensus 12H8 light chain variable region sequence (SEQ ID NOs: 3 and 4), respectively. The heavy chain CDR 1, 2 and 3 amino acid sequences are shown in SEQ ID NOs: 5, 6 and 7, respectively. The light chain CDR 1, 2 and 3 amino acid sequences are shown in SEQ ID NOs: 8, 9 and 10, respectively.

The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof, and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

CLAIMS

1. A Tie2 receptor antagonist antibody having the identifying characteristics of monoclonal antibody 12H8 or 6A3.
2. The antibody of claim 1 which is monoclonal antibody 12H8.
3. The antibody of claim 1 which is monoclonal antibody 6A3.
4. A polypeptide comprising a complementarity determining region (CDR) of the antibody of claim 2.
5. A polypeptide comprising a CDR of the antibody of claim 3.
6. An isolated polynucleotide encoding a polypeptide of claim 4 or 5.
7. An altered antibody comprising a heavy chain and a light chain, wherein the framework regions of said heavy and light chains are derived from at least one selected antibody and the amino acid sequences of the complementarity determining regions of each said chain are derived from monoclonal antibody 12H8 or 6A3.
8. The altered antibody of claim 7 which is humanized.
9. The altered antibody of claim 7 which is human.
10. An antibody comprising a heavy chain variable region polypeptide as set forth in SEQ ID NO: 2 and a light chain variable region polypeptide as set forth in SEQ ID NO: 4.
11. An isolated polynucleotide encoding the polypeptide of SEQ ID NO: 2 or SEQ ID NO: 4.
12. A heavy chain CDR having an amino acid sequence as set forth in SEQ ID NO: 5, 6 or 7.
13. An isolated polynucleotide encoding the CDR of claim 12.

14. A light chain CDR having an amino acid sequence as set forth in SEQ ID NO: 8, 9 or 10.
15. An isolated polynucleotide encoding the CDR of claim 14.
16. A pharmaceutical composition comprising the antibody of claim 1 or 7 and a pharmaceutically acceptable carrier.
17. A pharmaceutical composition comprising the polypeptide of claim 4 or 5 and a pharmaceutically acceptable carrier.
18. A hybridoma having the identifying characteristics of cell line 15B8 or 13H10.
19. The hybridoma of claim 18 which is cell line 12H8.
20. The hybridoma of claim 18 which is cell line 6A3.
21. A method for inhibiting angiogenesis in a mammal comprising administering an effective dose of a Tie2 receptor antagonist antibody having the identifying characteristics of monoclonal antibody 12H8 or 6A3.
22. The method of claim 21 wherein the subject is in need of treatment for angiogenic diseases.
23. The method of claim 21 wherein the angiogenic disease is diabetic retinopathy or macular degeneration.
24. The method of claim 21 wherein the subject is in need of treatment for proliferative diseases.
25. The method of claim 24 wherein the proliferative disease is cancer, arthritis, psoriasis or atherosclerosis.
26. A method for expansion of marrow cells transfected with a gene for gene therapy comprising treating the marrow cells with a Tie2 receptor antagonist antibody.

27. A method for expansion of cord blood, marrow or peripheral blood cells for transplant comprising treating the cells with a Tie2 receptor antagonist antibody.

28. A method to enhance the survival of hematopoietic progenitor cells in a mammal comprising treating the cells with a Tie2 receptor antagonist antibody.

29. A method to increase the proliferation of megakaryocyte cells or megakaryocyte progenitor cells to increase platelet numbers in a mammal comprising treating the cells with a Tie2 receptor antagonist antibody.

30. The method of claim 26, 27, 28 or 29 wherein the antibody has the identifying characteristics of monoclonal antibody 12H8 or 6A3.

SEQUENCE LISTING

<110> Connie L. Erickson-Miller
 Stephen D. Holmes
 James D. Winkler

<120> Tie2 Antagonist Antibodies

<130> P50844

<150> 60/102,100

<151> 1998-09-28

<160> 10

<170> FastSEQ for Windows Version 3.0

<210> 1

<211> 357

<212> DNA

<213> Mus musculus

<220>

<221> CDS

<222> (1)...(357)

<223> 12H8 heavy chain v region

<400> 1

gaa gtg caa ctt gta gag tct ggg gga ggc tta gtg aag cct gga ggg

48

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys Pro Gly Gly

1

5

10

15

tcc ctg aaa ctc tcc tgt gca gcc tct gga ttc act ttc agt gac tat

96

Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asp Tyr

20

25

30

gga atg cac tgg gtt cgt cag gct cca gag aag gga ctg gag tgg gtt

144

Gly Met His Trp Val Arg Gln Ala Pro Glu Lys Gly Leu Glu Trp Val

35

40

45

gca tac att aat agt ggc agt agc acc atc acc tat gca gac aca gtg
192

Ala Tyr Ile Asn Ser Gly Ser Ser Thr Ile Thr Tyr Ala Asp Thr Val
50 55 60

aag ggc cga ttc acc atc tcc aga gac aat gcc aag aac acc ctg ttc
240

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Phe
65 70 75 80

ctg caa atg acc agt ctg agg tct gag gac acg gcc ata tat tac tgt
288

Leu Gln Met Thr Ser Leu Arg Ser Glu Asp Thr Ala Ile Tyr Tyr Cys
85 90 95

gca agg ggc tac tac ggt ccg tac tac ttt gac tac tgg ggc caa ggc
336

Ala Arg Gly Tyr Tyr Gly Pro Tyr Tyr Phe Asp Tyr Trp Gly Gln Gly
100 105 110

acc gct ctc aca gtc tcc tca
357

Thr Ala Leu Thr Val Ser Ser
115

<210> 2

<211> 119

<212> PRT

<213> Mus musculus

<400> 2

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Lys Pro Gly Gly
1 5 10 15

Ser Leu Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Asp Tyr
20 25 30

Gly Met His Trp Val Arg Gln Ala Pro Glu Lys Gly Leu Glu Trp Val
35 40 45

Ala Tyr Ile Asn Ser Gly Ser Ser Thr Ile Thr Tyr Ala Asp Thr Val
50 55 60

Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Phe

```

65              70              75              80
Leu Gln Met Thr Ser Leu Arg Ser Glu Asp Thr Ala Ile Tyr Tyr Cys
              85              90              95
Ala Arg Gly Tyr Tyr Gly Pro Tyr Tyr Phe Asp Tyr Trp Gly Gln Gly
              100              105              110
Thr Ala Leu Thr Val Ser Ser
              115

```

```

<210> 3
<211> 321
<212> DNA
<213> Mus musculus

<220>
<221> CDS
<222> (1)...(321)
<223> 12H8 light chain v region

```

```

<400> 3
gat att gtt atg acg cag tct cac aaa ttc atg tcc aca tca gtg gga
48
Asp Ile Val Met Thr Gln Ser His Lys Phe Met Ser Thr Ser Val Gly
  1              5              10              15

gac agg gtc agc ttc acc tgc aag gcc agt cag aat gtg ggt act gct
96
Asp Arg Val Ser Phe Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Ala
      20              25              30

gta gcc tgg tat caa cag aaa cca ggt caa tct cct aaa cta ctg att
144
Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile
      35              40              45

tat tgg gca tcc tcc cgg cac act gga gtc cct gat cgc ttc aca ggc
192
Tyr Trp Ala Ser Ser Arg His Thr Gly Val Pro Asp Arg Phe Thr Gly
      50              55              60

agt gga tct ggg aca gat ttc act ctc acc att act aat gtg cag tct
240
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Thr Asn Val Gln Ser
      65              70              75              80

```

gaa gac ttg gca gat tat ttc tgt cag gaa tat agc agc tat cct ctc
288

Glu Asp Leu Ala Asp Tyr Phe Cys Gln Glu Tyr Ser Ser Tyr Pro Leu
85 90 95

acg ttc ggt gtt ggg acc aag ctg gag ctg aaa
321

Thr Phe Gly Val Gly Thr Lys Leu Glu Leu Lys
100 105

<210> 4

<211> 107

<212> PRT

<213> Mus musculus

<400> 4

Asp Ile Val Met Thr Gln Ser His Lys Phe Met Ser Thr Ser Val Gly
1 5 10 15
Asp Arg Val Ser Phe Thr Cys Lys Ala Ser Gln Asn Val Gly Thr Ala
20 25 30
Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile
35 40 45
Tyr Trp Ala Ser Ser Arg His Thr Gly Val Pro Asp Arg Phe Thr Gly
50 55 60
Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Thr Asn Val Gln Ser
65 70 75 80
Glu Asp Leu Ala Asp Tyr Phe Cys Gln Glu Tyr Ser Ser Tyr Pro Leu
85 90 95
Thr Phe Gly Val Gly Thr Lys Leu Glu Leu Lys
100 105

<210> 5

<211> 5

<212> PRT

<213> Mus musculus

<220>

<221> SITE

<222> (1)...(5)

<223> heavy chain CDR 1

```

<400> 5
Asp Tyr Gly Met His
1             5

<210> 6
<211> 17
<212> PRT
<213> Mus musculus

<220>
<221> SITE
<222> (1)...(17)
<223> heavy chain CDR 2

<400> 6
Tyr Ile Asn Ser Gly Ser Ser Thr Ile Thr Tyr Ala Asp Thr Val Lys
1             5             10             15
Gly

<210> 7
<211> 10
<212> PRT
<213> Mus musculus

<220>
<221> SITE
<222> (1)...(10)
<223> heavy chain CDR 3

<400> 7
Gly Tyr Tyr Gly Pro Tyr Tyr Phe Asp Tyr
1             5             10

<210> 8
<211> 11
<212> PRT
<213> Mus musculus

<220>
<221> SITE
<222> (1)...(11)
<223> light chain CDR 1

```

<400> 8
Lys Ala Ser Gln Asn Val Gly Thr Ala Val Ala
1 5 10

<210> 9
<211> 7
<212> PRT
<213> Mus musculus

<220>
<221> SITE
<222> (1)...(7)
<223> light chain CDR 2

<400> 9
Trp Ala Ser Ser Arg His Thr
1 5

<210> 10
<211> 9
<212> PRT
<213> Mus musculus

<220>
<221> SITE
<222> (1)...(9)
<223> light chain CDR 3

<400> 10
Gln Glu Tyr Ser Ser Tyr Pro Leu Thr
1 5

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/22421

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 39/395; C07K 16/28; C12N 15/13
US CL : 530/300, 387.1, 387.3, 388.22; 536/23.1, 23.5; 424/133.1, 143.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 530/300, 387.1, 387.3, 388.22; 536/23.1, 23.5; 424/133.1, 143.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MONESTIER ET AL. Molecular analysis of mercury-induced antinuclear antibodies in H-2s mice. J. Immunol. 1994, Vol. 152, pages 667-675, especially page 672.	4, 6, 14, 15, 17
X	TILLMAN ET AL. Both IgM and IgG anti-DNA antibodies are the products of clonally selective B cell stimulation in (NZB x NZW)F1 mice. J. Exp. Med. September 1992, Vol. 176, pages 761-779, especially page 766.	4, 6, 12, 13, 17
A	WO 95/21866 A1 (LUDWIG INSTITUTE FOR CANCER RESEARCH) 17 August 1995, see entire document.	1-25

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	*T
"A" document defining the general state of the art which is not considered to be of particular relevance	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

Date of mailing of the international search report

10 February 2000 (10.02.2000)

Authorized officer 29 FEB 2000

Name and mailing address of the ISA/US
Communications of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Claire M Kaufman

Facsimile No. (703) 305-3230

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/22421

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-25

Remark on Protest

☐
☐

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/22421

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-25, drawn to antibody and derivative, polynucleotide encoding, hybridoma and method of inhibiting angiogenesis.

Group II, claim(s) 26-30, drawn to method of expanding marrow cells with an antibody.

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Pursuant to 37 C.F.R. 1.475(d), this Authority considers that the main invention in the instant application comprises the first-recited product, TIE2 receptor antagonist antibodies and related products and the first-recited method of using that product, namely in the method of inhibiting angiogenesis with the antibody. Note that there is no method of making the antibody. Further, pursuant to 37 C.F.R. 1.475(b)-(d), the ISA/US considers that the additional method of Group II does not correspond to the main invention. This Authority therefore considers that the several inventions do not share a special technical feature within the meaning of PCT Rule 13.2 and thus do not relate to a single general inventive concept within the meaning of PCT Rule 13.1.

Continuation of B. FIELDS SEARCHED Item 3: Medline, Caplus, Inpadoc, WEST

search terms: tie2 or tie-2, receptor, antagonist, inhibit, antibody, monoclon, mab, s holmes, erickson, erickson-miller, connie miller, j winkler